

**Application
for
United States Letters Patent**

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To all whom it may concern:

Be it known that we,

Thomas M. Jessell, Konrad Basler and Toshiya Yamada

have invented certain new and useful improvements in

CLONING, EXPRESSION AND USES OF DORSALIN-1

of which the following is a full, clear and exact description.

CLONING, EXPRESSION AND USES OF DORSALIN-1

Ins a)

Background of the Invention

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Throughout this application various publications are referenced by the names of the authors and the year of the publication within parentheses. Full citations for these publications may be found at the end of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

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Inductive interactions that define the fate of cells within the neural tube establish the initial pattern of the embryonic vertebrate nervous system. In the spinal cord, the identity of cell types is controlled, in part, by signals from two midline cell groups, the notochord and floor plate which induce neural plate cells to differentiate into floor plate, motor neurons and other ventral neuronal types (van Straaten et al. 1988; Placzek et al. 1990, 1993; Yamada et al. 1991; Hatta et al. 1991). The induction of floor plate cells appears to require a contact-mediated signal (Placzek et al. 1990a, 1993) whereas motor neurons can be induced by diffusible factors (Yamada et al., 1993). Thus, the fate of different ventral cell types may be controlled by distinct signals that derive from the ventral midline of the neural tube.

The specification of dorsal cell fates appears not to require ventral midline signals since the neural tube still gives rise to dorsal cell types such as sensory relay neurons and neural crest cells after elimination of

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the notochord and floor plate (Yamada et al. 1991; Placzek et al. 1991; Ericson et al. 1992). Moreover, dorsal cell types are found at more ventral positions in such embryos (Yamada et al. 1991; Placzek et al. 1991) suggesting that many or all cells in neural tube have acquired dorsal characteristics. The acquisition of a dorsal fate could represent a default pathway in the differentiation of neural plate cells or a response to inductive factors that are distinct from the ventralizing signals that derive from the notochord and floor plate.

To identify signals that might regulate cell differentiation within the neural tube, genes encoding secreted factors that are expressed in a restricted manner along the dorsoventral axis of the neural tube have been searched. In this application, the transforming growth factor β (TGF β) family have been focused since some of its members have been implicated in the control of cell differentiation and patterning in non-neural tissues. In frog embryos, for example, the differentiation and patterning of mesodermal cell types appears to be controlled, in part, by the action of activin-like molecules (Ruiz i Altaba and Melton, 1989; Green and Smith, 1990; Thomsen et al. 1990; Green et al. 1992). In addition, the dorsoventral patterning of cell types in *Drosophila* embryos is regulated by the *decapentaplegic* (*dpp*) gene (Ferguson and Anderson, 1992a,b). The *dpp* protein is closely related to a subgroup of vertebrate TGF β -like molecules, the bone morphogenetic proteins (BMPs) (Wozney et al. 1988), several members of which are expressed in restricted regions of the developing embryos (Jones et al. 1991).

In this application, the cloning and functional characterization of the *dorsalin-1* (*dsl-1*) gene, which

encodes a novel BMP-like member of the TGF- β superfamily
are described. *Dsl-1* is expressed selectively by cells
in the dorsal region of the neural tube and its
expression in ventral regions appears to be inhibited by
5 signals from the notochord. *Dsl-1* promotes the
differentiation or migration of neural crest cells and
can prevent the differentiation of motor neurons in
neural plate explants. The combined actions of *dsl-1* and
ventralizing factors from the notochord and floor plate
10 may regulate the identity of neural cell types and their
position along the dorsoventral axis of the neural tube.

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Summary of the Invention

5 This invention provides an isolated vertebrate nucleic acid molecule which encodes dorsalin-1. This invention also provides a nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence included within the sequence of a nucleic acid molecule encoding a dorsalin-1.

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The invention provides a vector which comprises an isolated nucleic acid molecule of dorsalin-1 operatively linked to a promoter of RNA transcription. This invention further provides a host vector system for the production of a polypeptide having the biological activity of dorsalin-1 which comprises the above-described vector in a suitable host.

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This invention also provides a method of producing a polypeptide having the biological activity of dorsalin-1 which comprises growing the above-described host vector system under suitable conditions permitting production of the polypeptide and recovering the polypeptide so produced.

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This invention also provides a purified vertebrate dorsalin-1. This invention further provides a purified human dorsalin-1.

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This invention provides a method for stimulating neural crest cell differentiation in a subject comprising administering to the subject an amount of a purified dorsalin-1 effective to stimulate neural crest cell differentiation.

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This invention provides a method for regenerating nerve cells in a subject comprising administering to the subject an amount of a purified dorsalin-1 effective to regenerate nerve cells.

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This invention provides a method for promoting bone growth in a subject comprising administering to the subject an amount of a purified dorsalin-1 effective to promote bone growth.

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This invention provides a method for promoting wound healing in a subject comprising administering to the subject an amount of a purified dorsalin-1 effective to promote wound healing.

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This invention provides a method for treating neural tumor in a subject comprising administering to the subject an amount of a purified dorsalin-1 effective to inhibit the tumor cell growth.

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This invention further provides a pharmaceutical composition for stimulating neural crest cell differentiation comprising an amount of a purified dorsalin-1 effective to stimulate neural crest cell differentiation and a pharmaceutically acceptable carrier.

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This invention provides a pharmaceutical composition for regenerating nerve cells in a subject comprising an amount of a purified dorsalin-1 effective to regenerate nerve cells and a pharmaceutically acceptable carrier.

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This invention provides a pharmaceutical composition for promoting bone growth in a subject comprising an amount of a purified dorsalin-1 effective to promote bone growth

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and a pharmaceutically acceptable carrier.

5 This invention provides a pharmaceutical composition for promoting wound healing in a subject comprising an amount of a purified dorsalin-1 effective to promote wound healing and a pharmaceutically acceptable carrier.

10 This invention provides a pharmaceutical composition for treating neural tumor in a subject comprising an amount of a purified dorsalin-1 effective to inhibit neural tumor cell growth and a pharmaceutically acceptable carrier.

15 This invention provides an antibody capable of binding to dorsalin-1. This invention also provides an antibody capable of inhibiting the biological activity of dorsalin-1.

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Brief Description of Figures

Figure 1

Nucleotide and Deduced Amino Acid Sequence of Dorsalin-1 (SEQ. ID No. 1.)

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The numbering of the protein sequence starts with the first methionine of the long open reading frame. The putative signal sequence is typed in bold letters. The RSKR (SEQ. ID No. 17) sequence preceding the proteolytic cleavage site (arrow) is underlined. The site of insertion of the 10 amino acid c-myc epitope is marked with an asterisk. The accession number for dorsalin-1 is L12032.

Figure 2

Dorsalin-1 is a Member of the TGF- β Superfamily

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(A) Alignment of the COOH-terminal amino acid sequences of dorsalin-1 and some representative members of the TGF- β superfamily. Residues that are identical in at least 4 of the 7 proteins are printed in white on a black background. The 7 conserved cysteine residues are marked with an asterisk. Gaps introduced to optimize the alignment are represented by dashes. Known proteolytic cleavage sites in these proteins are marked with an arrow head. Numbers at the right indicate the number of amino acids present in the protein.

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(B) Graphical representation of the

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sequence relationship between members of the TGF- β superfamily. This tree representation has been generated using the program pileup of the GCG software package (Devereux et al., 1984). Underneath each branch the percentage amino acid identity is shown with reference to dorsalin-1. This value was calculated using the local homology algorithm of Smith and Waterman (1981) implemented in the program bestfit (GCG software). For both the tree and the amino acid identities only the sequence of the COOH-terminal domain was used, starting with the first of the seven conserved cysteine residues and ending with COOH-terminal residue. For details of other TGF- β family members see Lee (1990), Lyons et al. (1991), Hoffmann, (1991).

Figure 3

Affinity Purification and Functional Activity of Recombinant Dorsalin-1 Protein

(A) Dorsalin-1^{myc} protein was purified from cos-7 cell-conditioned medium using a MAb 9E10 affinity column. An aliquot of the purified protein (CM) was run on a 15% SDS-polyacrylamide gel and stained with Coomassie Blue. The arrow points to the major product running at a molecular weight of ~15 kDa and minor bands at 45, 47 and 60 kDa are also evident. NH₂-terminal sequencing of the 15 kDa band confirmed its identity as processed

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dorsalin-1^{myc} protein. Affinity-purified conditioned medium obtained from mock-transfected cos-7 cells did not contain any detectable protein on a Coomassie Blue stained acrylamide gel (not shown). The positions of molecular weight standards (MW) are shown.

(B) Induction of Alkaline Phosphatase Activity in W-20-17 Cells by Dorsalin-1. Conditioned medium was harvested from cos-7 transfected with *dsl-1* cDNA, with the *dsl-1^{myc}* cDNA and added at different dilutions to W20-17 cells for 72h and alkaline phosphatase activity assayed (Thies et al. 1992). As a control for the presence of BMP-like activity in cos-7 cells, medium was also obtained from cells transfected with a c-myc tagged construct encoding the *Drosophila decapentaplegic (dpp)* gene, a related TGF β family member since (see Fig. 2B). *Dpp^{myc}* is not detectable in the medium of transfected cos-7 cells. Curves are from one of three experiments that produced similar results. Recombinant human BMP-2 (Thies et al. 1992) was used on a positive control in the assay.

Figure 4

Dorsalin-1 mRNA expression in the embryonic chick spinal cord

Panels represent pairs of phase-contrast and dark-field micrographs of sections of embryonic chick neural tube and spinal

cord, processed for localization of *dorsalin-1* mRNA by in situ hybridization with ³⁵S-labelled probe.

- 5 (A,B) *Dorsalin-1* mRNA is not expressed in neural cells at stages before neural tube closure. The dark field micrograph (B) shows background grain densities.
- 10 (C,D) *Dorsalin-1* mRNA is expressed at high levels in the dorsal third of the neural tube, beginning at the time of neural tube closure, but not by ventral neural cells or by non-neural cells. This section is
- 15 taken from a HH stage 10 embryo at the future brachial level.
- 20 (E,F) The dorsal restriction of *dorsalin-1* mRNA persists in the spinal cord at stages after the onset of neuronal differentiation. Section taken from HH stage 22 embryo, at the brachial level.
- 25 (G,H) At later stages of spinal cord development (HH St 26) *dorsalin-1* mRNA is restricted to the dorsomedial region of the spinal cord, including but not confined to the roof plate.
- 30 Scale bar: A,B=35 μ m, C-F=80 μ m, G-H=140 μ m.

Figure 5

Regulation of *dorsalin-1* mRNA expression by notochord

(A,B) Phase-contrast and dark-field images of a section of spinal cord from an operated stage 22 embryo but at a level in which there is no grafted tissue. The pattern of *dorsalin-1* mRNA expression is similar to that in unoperated embryos at the same developmental age.

(C) Phase-contrast micrograph section from an embryo at the same stage as that shown in A,B, showing the expression of SC1 by motor neurons and floor plate cells, detected by immunoperoxidase histochemistry.

(D,E) Phase-contrast and dark-field images of a section of spinal cord from an operated stage 22 embryo in which there is a dorsally-located notochord (n). The expression of *dorsalin-1* RNA is suppressed in the presence of a dorsal notochord graft. Similar results were obtained in 2 other embryos.

(F) Phase-contrast micrograph of an adjacent section to that shown in D,E, showing the ectopic dorsal location of SC1⁺ motor neurons that form a bilaterally symmetric continuous column. SC1⁺ motor axons can be seen leaving the dorsal spinal cord.

SC1⁺ floor plate cells are detected at the dorsal midline. The position of the grafted notochord is indicated (n').

(G,H) Phase-contrast and dark-field micrographs showing that *dorsalin-1* mRNA expression expands to occupy the entire neural epithelium in embryos from which Hensen's node has been removed at HH stage 10. In this embryo the operation resulted in a splitting of the neural tube and this micrograph has been spliced to restore the ventral apposition of neural tissue. Splitting of the neural tube occurs frequently after removal of Hensen's node (Darnell et al. 1992). A partial or complete ventral expansion of *dsl-1* expression was detected in a total of 5 embryos with Hensen's node removal. A ventral expression of *dsl-1* expression, occupying 60-70% of the spinal cord was also detected after notochord removal in 2 embryos.

Scale bar: A-F=90 μ m, G-H=45 μ m.

Figure 6

Induction of Cell Migration from [i]-Neural Plate Explants by Dorsalin-1

[i]-Neural plate explants were grown alone or in the presence of *dsl-1*^{myc} (3×10^{-11} M) 48h, and migratory cells analyzed by phase-contrast microscopy and by expression of surface antigens.

(A) Phase contrast micrograph of [i]-neural plate explant grown alone for 48h.

(B) Phase contrast micrograph of [i]-

5 (C) Phase contrast micrograph of an [i]-
neural plate explant grown in contact with
notochord (n) in the presence of dsl-1^{myc}
for 48h. Cells still emigrate from the
10 explant although few cells are located in
the vicinity of the notochord explant.

(D) Expression of HNK-1 by cells induced
to migrate from [i]-neural plate explant
by dsl-1^{myc}.

15 (E) Expression of β 1-integrin by cells
induced to emigrate from [i]-neural plate
explant. About 30% of migratory cells
expressed p75, although the levels
20 appeared lower than that detected on
neural crest cells derived from the dorsal
neural tube.

(F) Expression of melanin by cells induced
to migrate from quail [i]-neural plate
25 explants by dsl-1^{myc}. In these
experiments dsl-1^{myc} was removed from
after 48h and cultures grown in the
presence of chick embryo extract (CEE) for
30 a further 72h. About 10-15% of cells in
this bright field micrograph exhibit
melanin pigment and typical dendritic
morphology. Two different focal planes of
the same field are shown to maintain
35 melanocytes in focus. Similar results

were obtained in 6-8 explants tested. For details see text.

(G) Quantitation of cell migration induced by *dsl-1*. [i]np indicates [i]-neural plate explant. nc=notochord, fp=floor plate. Error bars represent the means \pm s.e.m. of migrated cells for 10-26 different explants.

Scale bar: A-C=70 μ m, D-F=35 μ m.

Figure 7

Induction of Islet-1 expression in neural plate explants and suppression by *dorsalin-1*

(A-C) Nomarski (A) and immunofluorescence (B,C) micrographs of stage 9-10 chick [i]-neural plate explant grown for 48h in the absence of notochord or floor plate. Islet-1⁺ cells are not detected (B) but there is extensive neuronal differentiation as detected by 3A10 expression (C).

(D-F) Nomarski (D) and immunofluorescence (E,F) micrographs of [i]-neural plate explant grown in contact with stage 26 chick floor plate. Numerous Islet-1⁺ cells are present in the [i]-neural plate explant (np), but not in the floor plate explant (fp). The explant also contains many 3A10⁺ cells (F).

(G-I) Nomarski (G) and immunofluorescence

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micrographs (H,I) of [i]-neural plate explant exposed for 48h to floor plate-conditioned medium. Numerous Islet-1⁺ cells (H) and 3A10⁺ neurons (I) are detected.

(J-L) Nomarski (J) and immunofluorescence micrograph (K,L) of an [i]-neural plate and floor plate conjugate exposed for 48h to $3 \times 10^{-11} \text{M}$ dorsalin-1^{myc}. No Islet-1⁺ cells are detected (K) whereas the number of 3A10⁺ neurons in the neural plate explant (L) is not obviously different from that in the absence of dorsalin-1^{myc}. In figures D and G, the dashed line outlines the extent of the neural plate (np) explant.

Scale bar: A-C=70 μm , D-F=100 μm , G-I=70 μm , J-L=100 μm .

Figure 8

Inhibition of Islet-1⁺ Cells by Dorsalin-1

(A) Histograms showing the induction of Islet-1⁺ cells in [i]-neural plate explants by contact with notochord (nc) or floor plate (fp), and the inhibition of Islet-1⁺ cells by dorsalin-1^{myc} ($3 \times 10^{-11} \text{M}$). Each column represents mean \pm s.e.m. of 10-22 different explants.

(B) Dose-dependent inhibition of Islet-1⁺ cells by dorsalin-1^{myc}. Each point represents mean \pm s.e.m. of 7-23 different explants.

(C) Induction of Islet-1⁺ cells by floor plat -conditioned medium and the inhibitory action of dorsalin-1^{myc}. Each column represents mean \pm s.e.m. of 7-23 explants.

[i]np=[i]-neural plate explant grown alone, +nc=neural plate/notochord conjugate, +fp=neural plate/floor plate conjugate, fpcm=floor plate-conditioned medium.

Figure 9

Potential Functions of Dorsalin-1 in the Control of Cell Differentiation in the Neural Tube

Diagrams summarize the possible mechanisms for establishing the dorsally-restricted expression of dorsalin-1 and potential functions of dorsalin-1 in the regulation of cell differentiation along the dorsoventral axis of the neural tube.

(A) The pattern dorsalin-1 expression appears to be established by early signals from the notochord. (i) Medial neural plate cells respond to signals from the underlying notochord which induce the differentiation of ventral cell types such as floor plate and motor neurons. (ii) Medial neural plate cells are also exposed to signals from the notochord that prevent the subsequent expression of dorsalin-1. The inhibitory signal from the notochord can, in principle, be identical to the

ventralizing signal that induces ventral cell fates. (iii) The medial region of the neural plate gives rise to the ventral neural tube. *Dorsalin-1* expression (shaded area) begins at the time of neural tube closure and is restricted to the dorsal third of the neural tube.

(B) In vitro assays suggest several possible functions for *dorsalin-1* in the control of neural cell differentiation.

(i) *Dorsalin-1* may promote the differentiation of cell types that derive from the dorsal region of the neural tube.

In vitro studies suggest that neural crest cells represent one population of cells whose differentiation may be influenced by *dorsalin-1*. (ii) The dorsal expression of *dorsalin-1* may define the dorsal third of the neural tube as a domain that is refractory to the long range influence of ventralizing signals from the notochord and floor plate. The ventral boundary of *dorsalin-1* expression suggests that

ventral midline-derived signals can influence cells over much of the dorsoventral axis of the neural tube.

(iii) *Dorsalin-1* protein may diffuse ventrally to influence the fate of cells in intermediate regions of the neural tube beyond the domain of *dorsalin-1* mRNA expression. Thus, the combined action of *dorsalin-1* and the diffusible ventralizing signal from the notochord and floor plate could specify the fate of cells over the

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complete dorsoventral axis of the neural tube.

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Figure 10

~~Amino acid comparison of chick dorsalin-1 (B29) and mouse (B29m).~~

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Detailed Description of the Invention

5 This invention provides an isolated vertebrate nucleic acid molecule encoding dorsalin-1. As used herein, the term dorsalin-1 encompasses any amino acid sequence, polypeptide or protein having the biological activities provided by dorsalin-1.

10 In one embodiment of this invention, the isolated nucleic acid molecules described hereinabove are DNA. In a further embodiment, isolated nucleic acid molecules described hereinabove are cDNAs or genomic DNAs. In the preferred embodiment of this invention, the isolated nucleic sequence is cDNA as shown in sequence ID number
15 1. In another embodiment, the isolated nucleic acid molecule is RNA.

20 This invention also encompasses DNAs and cDNAs which encode amino acid sequences which differ from those of dorsalin-1, but which should not produce phenotypic changes. Alternatively, this invention also encompasses DNAs and cDNAs which hybridize to the DNA and cDNA of the subject invention. Hybridization methods are well-known to those of skill in the art.

25 The DNA molecules of the subject invention also include DNA molecules coding for polypeptide analogs, fragments or derivatives of antigenic polypeptides which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues (deletion
30 analogs containing less than all of the residues specified for the protein, substitution analogs wherein one or more residues specified are replaced by other residues and addition analogs where in one or more amino
35 acid residues is added to a terminal or medial portion of

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the polypeptides) and which share some or all properties of naturally-occurring forms. These molecules include: the incorporation of codons "preferred" for expression by selected non-mammalian host; the provision of sites for cleavage by restriction endonuclease enzymes; and the provision of additional initial, terminal or intermediate DNA sequences that facilitate construction of readily expressed vectors.

The DNA molecules described and claimed herein are useful for the information which they provide concerning the amino acid sequence of the polypeptide and as products for the large scale synthesis of the polypeptide by a variety of recombinant techniques. The molecules are useful for generating new cloning and expression vectors, transformed and transfected procaryotic and eucaryotic host cells, and new and useful methods for cultured growth of such host cells capable of expression of the polypeptide and related products.

Moreover, the isolated nucleic acid molecules are useful for the development of probes to study the neurodevelopment.

Dorsalin-1 may be produced by a variety of vertebrates. In an embodiment, a human dorsalin-1 nucleic acid molecule is isolated. In another embodiment, a mouse dorsalin-1 nucleic acid molecule is isolated. In a further embodiment, a chick dorsalin-1 nucleic acid molecule is provided. The plasmid, pKB502, encoding a chick dorsalin-1 was deposited on October 5, 1992 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism

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for the Purposes of Patent Procedure. Plasmid, pKB502 was accorded ATCC Accession number 75321.

5 Throughout this application, references to specific nucleotides are to nucleotides present on the coding strand of the nucleic acid. The following standard abbreviations are used throughout the specification to indicate specific nucleotides:

10 C=cytosine A=adenosine
T=thymidine G=guanosine

15 For the purpose of illustration only, applicants have isolated and characterized dorsalin-1 cDNA clones from chicken and mouse. Similar techniques are applicable to isolate and characterize the dorsalin-1 genes in different vertebrates.

20 Dorsalin-1 genes may be isolated using the probe generated from the chick dorsalin-1 gene. The mouse and human homologous genes may be cloned by using probe from the chick gene by low stringency screening of the correspondent embryonic spinal cord cDNA libraries. A mouse dorsalin-1 was cloned using the above method. Figure 10 shows a mouse homolog of the dorsalin-1 which
25 reveals extensive conservation at the nucleotide and amino acid level with the chick dorsalin-1. The human dorsalin-1 is likely to be more closely related to the mouse protein than is the chick protein. Thus, it should be straightforward to design oligonucleotide primers to
30 isolate the human dorsalin-1 gene.

35 This invention provides a nucleic acid molecule comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence included within the sequence of a nucleic acid

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This invention further provides an isolated DNA or cDNA molecule described hereinabove wherein the host cell is selected from the group consisting of bacterial cells (such as E.coli), yeast cells, fungal cells, insect cells and animal cells. Suitable animal cells include, but are not limited to Vero cells, HeLa cells, Cos cells, CV1 cells and various primary mammalian cells.

This invention provides a method to identify and purify expressed dorsalin-1. A myc-epitope was introduced into dorsalin-1. This myc carrying dorsalin-1 was linked to an expression vector. Such vector may be used to transfect cell and the distribution of dorsalin-1 in the cell may be detected by reacting myc antibodies known to be reactive to the introduced myc-epitope with the transfected cells which is expressing the dorsalin-1 carrying myc-epitope. Taking advantage of this myc-epitope, dorsalin-1 may be purified by an antibody affinity column which binds with this myc-epitope.

In one embodiment, the expression vector, pKB501 (with myc epitope), containing chick dorsalin-1 with a myc-epitope was deposited on October 5, 1992 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. Plasmid, pKB 501 (with myc epitope) was accorded ATCC designation number 75320.

The above uses of the myc-epitope for identification and purification of dorsalin-1 should not be considered limiting only to the myc-epitope. Other epitopes with specific antibodies against them which are well known to an ordinary skilled in the art could be similarly used.

Also provided by this invention is a purified vertebrate dorsalin-1. As used herein, the term "purified vertebrate dorsalin-1" shall mean isolated naturally-occurring dorsalin-1 or protein (purified from nature or manufactured such that the primary, secondary and tertiary conformation, and posttranslational modifications are identical to naturally-occurring material) as well as non-naturally occurring polypeptides having a primary structural conformation (i.e. continuous sequence of amino acid residues). Such polypeptides include derivatives and analogs. In one embodiment, the purified dorsalin-1 is human dorsalin-1.

This invention also provides polypeptides encoded by the above-described isolated vertebrate nucleic acid molecules.

This invention provides a method for stimulating neural crest cell differentiation in a culture comprising administering an amount of the above-described purified dorsalin-1 effective to stimulate neural crest cell differentiation to the culture.

This invention also provides a method for stimulating neural crest cell differentiation in a subject comprising administering to the subject an amount of the above-described purified dorsalin-1 effective to stimulate neural crest cell differentiation.

This invention provides a method for regenerating nerve cells in a subject comprising administering to the subject an effective amount of the above-described purified dorsalin-1 effective to regenerate nerve cells.

This invention provides a method for promoting bone

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growth in a subject comprising administering to the subject an effective amount of the above-described purified dorsalin-1 effective to promote bone growth.

5 This invention provides a method for promoting wound healing in a subject comprising administering to the subject an effective amount of above-described purified dorsalin-1 effective to promote wound healing.

10 This invention provides a method for treating neural tumor in a subject comprising administering to the subject an amount of the above-described purified dorsalin-1 effective to inhibit the tumor cell growth. In an embodiment, the neural tumor is neurofibroma. In
15 another embodiment, the neural tumor is Schwann cell tumor.

This invention also provides a method for preventing differentiation of motor neurons in a culture comprising
20 administering an amount of purified dorsalin-1 neurons to the culture.

This invention also provides a method for preventing differentiation of motor neurons in a subject comprising
25 administering to the subject an amount of the above-described dorsalin-1 effective to prevent differentiation of motor neurons.

This invention also provides a pharmaceutical composition
30 for stimulating neural crest cell differentiation comprising an amount of purified dorsalin-1 of claim 18 effective to stimulate neural crest cell differentiation and a pharmaceutically acceptable carrier.

35 As used herein, "pharmaceutically acceptable carriers"

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means any of the standard pharmaceutically acceptable carriers. Examples include, but are not limited to, phosphate buffered saline, physiological saline, water and emulsions, such as oil/water emulsions.

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This invention provides a pharmaceutical composition for regenerating nerve cells in a subject comprising an amount of the above-described purified dorsalin-1 effective to regenerate nerve cells and a pharmaceutically acceptable carrier.

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This invention provides a pharmaceutical composition for promoting bone growth in a subject comprising an amount of the above-described purified dorsalin-1 effective to promote bone growth and a pharmaceutically acceptable carrier.

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This invention provides a pharmaceutical composition for promoting wound healing in a subject comprising an amount of the above-described purified dorsalin-1 effective to promote wound healing and a pharmaceutically acceptable carrier.

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This invention provides a pharmaceutical composition for treating neural tumor in a subject comprising an amount of the above-described purified dorsalin-1 effective to inhibit neural tumor cell growth and a pharmaceutically acceptable carrier. In an embodiment of this pharmaceutical composition, the neural tumor is neurofibroma. In another embodiment of this pharmaceutical composition, the neural tumor is Schwann cell tumor.

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Also provided by this invention is a method to produce antibody using the above-described purified dorsalin-1.

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EXPERIMENTAL DETAILS

Experimental Procedures

5 RNA Isolation and PCR Amplification

Spinal cord tissue was dissected from 80 embryonic day (E) 2.5 chicks. Poly (A)⁺ RNA (20 µg) was isolated from this tissue using an oligo (dT)-cellulose spin column (Pharmacia®) and 1.5 µg was used in two first strand cDNA synthesis reactions with either oligo (dT) or random hexanucleotides as primers for the reverse transcriptase reaction. One third of each of the two cDNA reaction mixture was combined and used as template for PCR amplification using 100 pmoles of the following degenerate primers in a reaction volume of 50 µl:

10 5' TGGAATTCTGG (ACG) A (ACGT) GA (CT) TGGAT (ACT) (AG) T (ACGT) GC
3' (SEQ ID No. 10)

and

20 5' GAGGATCCA (AG) (ACGT) GT (CT) TG (ACGT) AC (AGT) AT (ACGT) GC (AG) TG
3' (SEQ ID No. 11)

where degenerate positions are in parenthesis and restriction sites underlined. These oligonucleotides correspond to the *dorsalin-1* amino acid positions 339-345 and 377-371, respectively. The reaction was cycled twice between 94° (50 seconds), 50° (2 minutes), and 72° (2 minutes), followed by 28 rounds of 94° (50 seconds), 55° (2 minutes), and 72° (1.5 minutes). The reaction products were purified, digested with BamHI and EcoRI, size selected by agarose gel electrophoresis and cloned into the bacteriophage vector M13mp18. 50 clones were picked randomly and analyzed on a sequencing gel by comparing their G ladders. One member of each class was sequenced completely.

DNA Isolation and Sequencing

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An E2.5 chick spinal cord cDNA library of 10^6 independent clones was constructed in lambda ZAPII (Stratagene®) using 5 µg of the poly(A)+ RNA described above. After amplifying the library, 10^6 clones were screened under standard hybridization conditions and a ^{32}P -labeled PCR probe derived from the 116 bp insert of M13 clone B29 representing the *dorsalin-1* class. Of approximately 25 positive clones, 4 were plaque-purified and converted into pBluescript plasmids. Sequence analysis was performed by a combination of primer walking and subcloning of small restriction fragments into M13. The sequence within and adjacent to the long open reading frame was determined on both strands by the dideoxy chain termination method (Sanger et al. 1977) using Sequenase (U.S. Biochemicals).

DNA Constructs

The coding region of *dorsalin-1* was isolated using the two PCR primers ORF-5' (5' TGAATTCATCGATAACGGAAGCTGAAGC 3'; SEQ ID No. 12) and ORF-3' (5' AGCGTCGACATCGATATTCAGCATATACTACC 3'; SEQ ID No. 13) and cloned into pBS SK-between the EcoRI and SalI sites. To insert the c-myc epitope (EQKLISEEDL; SEQ. ID No. 18) two internal primers, each encoding half of the c-myc epitope and *dorsalin* sequences from the epitope insertion site (see Figure 1), were used to produce two PCR fragments, one encoding *dorsalin* N-terminal to the insertion site (with primer ORF-5' and the primer 5' GCGAATTCGATATCAGCTTCTGCTCTGCTCCTATGCTTCTCTTGC 3' [SEQ. ID No. 14]) and the other encoding the C-terminal region (with primer 5' CGGAATTCGATATCCGAGGAGGACCTGAACCACTGTCGGAGAACGTC 3'; SEQ

ID No. 15 and primer ORF-3'). These two fragments were joined using their primer-derived EcoRV sites and cloned the same way as the unmodified coding region. Using nearby primers this region was sequenced to confirm that no other mutations had been introduced.

A truncated coding region was derived from this construct by cleavage with HindIII, blunting the ends with T4 DNA polymerase and subsequent religation. This leads to a frame-shift mutation which replaces the C-terminal 41 residues of *dorsalin* with 9 unrelated ones. The unmodified, the epitope-tagged and the truncated *dorsalin* coding regions were then cloned into the Cos-7 cell expression vector pMT21 between the EcoRI and XhoI sites.

In Situ Hybridization Histochemistry

A *dorsalin-1* cDNA clone was linearized with XbaI (at amino acid position 176) and used to generate a 1 kb [³⁵S]UTP-labeled antisense RNA probe using T7 RNA polymerase. This probe encompasses the 3' part of the cDNA. Chick embryos were fixed in 4% paraformaldehyde and 10 μm cryostat sections were mounted on 3-aminopropyltriethoxysilane-treated slides. In situ hybridization was performed essentially as described by Wilkinson, et al. (1987) with exposure times ranging from 4 to 10 days. The distribution of *dorsalin-1* mRNA was confirmed by whole-mount in situ hybridization, performed essentially as described by Harland (1991) using a digoxigenin-11-UTP-labeled RNA probe derived from the template mentioned above (not shown).

Chick Embryo Manipulations

Notochord grafting and deletion in ovo was performed as

described by Yamada et al. (1991). For removal of Hensen's node from stage 9-10 chick embryos in ovo, the embryo was visualized by injection of India ink underneath the cavity between the yolk and embryo. Hensen's node was cut out together with underlying endoderm using fine tungsten needles. After the operation, the window was sealed and the embryo was incubated for further 48h at 37°C in the humidified incubator. Embryos were then fixed with 4% paraformaldehyde overnight at 4°C and embedded in paraffin for in situ hybridization as described above.

Cos-7 Cell Transfections

Cos-7 cells were transfected by the DEAE-Dextran method as described by Klar, et al. 1992). For small scale cultures 60 to 100 µm dishes were used and conditioned medium was prepared by incubating cells expressing dorsalin-1 for 48h in 3 or 6 ml of OPTI-MEM (BRL®), respectively. Large-scale transfections for affinity-purification of dorsalin-1 comprised 15 x 150 mm dishes for transfection with dorsalin^{myc} DNA (bearing the myc epitope) and an equal number of dpp or mock-transfected plates. This yielded 150 ml of dorsalin^{myc} conditioned medium and 150 ml of cos-7 conditioned control medium. The BMP-4 expression plasmids was provided by R. Derynck.

Affinity Purification and Sequence Analysis of dorsalin-1^{myc}

Conditioned medium (50 ml) containing dsl-1^{myc} was clarified by centrifugation at 30,000 x g and affinity-purified on 1 ml of a monoclonal 9E10 (anti-myc) antibody column (Affi-Gel, Biorad®). Dsl-1^{myc} protein was eluted with 0.1 M glycine-HCl (pH 2.5) and immediately

neutralized with 3 M Tris base. The eluate was concentrated and desalted over a 2 ml Centricon-10 microconcentrator (Amicon). The protein concentration of the final fraction (volume approximately 130 μ l), as
5 determined by amino acid analysis, was 0.1 μ g/ml.

For SDS-polyacrylamide gel electrophoresis, 10 μ l of concentrated protein was loaded on a 15% Biorad Mini-Protean II gel and stained with Coomassie Blue. 60 μ l
10 was used on a preparative gel and blotted onto Immobilon membrane in the absence of glycine. The blot was stained briefly with Coomassie Blue and the major band at 15 kD was excised and subjected to N-terminal protein sequencing on a Applied Biosystems 470A gas phase
15 sequencer/120A PTH analyzer. The minor protein migrating slightly slower on the gel (at 16.5 kD) was also sequenced and had the identical N-terminus, suggesting that it is an alternately glycosylated form of *dsl-1*. Affinity-purified conditioned medium from mock-
20 transfected cos-7 cells did not contain any detectable protein on a Coomassie-stained acrylamide gel.

The concentration of *dorsalin-1^{myc}* used for bioassays was determined on the assumption that all activity resides in
25 the ~15 kDa band which represents about 50% of the protein recovered after affinity-purification. The total protein in the affinity-purified fraction determined by amino acid analysis was found to be 100 ng/ μ l, of which 50 ng/ μ l is assumed to represent active protein. The
30 stock concentration of *Dsl-1^{myc}* was therefore 3×10^{-6} M. This stock was then diluted 10^5 fold for most assays to give a final condition of 3×10^{-11} M, assuming negligible losses.

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Islet-1 Induction Assay

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5 The assay for induction of Islet-1+ cells was carried out
as described in detail in Yamada et al. 1993. [i]-Neural
plate explants were isolated from Hamburger Hamilton HH
stage 10 chick embryos (Yamada et al. 1993) and grown in
collagen gels alone or with HH stage 10 notochord, HH
stage 26 floor plate or with floor plate-conditioned
10 medium in F12-N3 defined culture medium (Tessier-Lavigne
et al. 1988) at 37°C for 48 to 120h. Floor plate-
conditioned medium was obtained by culturing 30 HH stage
25-26 floor plate fragments in 1 ml of F12 N3 medium for
48h.

15 After incubation, explants were fixed with 4%
paraformaldehyde at 4°C for 1-2h, washed with PBS at 4°C
and gently peeled from the bottom of the dish and excess
collagen gel was trimmed. Explants were incubated with
20 primary antibodies overnight at 4°C with gentle
agitation. Rabbit anti-Islet-1 antibodies (Thor et al.
1991, Ericson et al. 1992) and MAb SC1 (Tanaka and Obata,
1984) were used for detection of differentiating motor
neurons and MAb 3A10 as a general neuronal marker (Dodd
25 et al., 1988). After washing with PBS for 2h at 22°C,
the explants were incubated with Texas Red conjugated
goat anti-rabbit antibodies (Molecular Probes) or FITC-
conjugated goat anti-mouse Ig (Boehringer Mannheim) for
1-2h. Explants were washed with PBS at 22°C for 2h with
30 at least two changes of buffer and mounted on slides in
50% glycerol with paraphenylene diamine (1 mg/ml). The
number of Islet-1⁺ and 3A10⁺ cells was determined on a
Zeiss Axiophot microscope equipped with epifluorescence
optics. Double labeling with anti-Islet-1 and anti-SCI
35 antibodies was analyzed using BioRad confocal microscope.

Analysis of Neural Crest Differentiation

[i]-Neural plate explants from stage 10 chick embryos were grown in collagen gels as described for analysis of Islet-1 induction. The number of migratory cells was determined by phase-contrast microscopy. Cells were scored as migratory if they were greater than two cell body diameters away from the mass of the [i]-neural plate explant. Identification of surface antigens was performed on cultures fixed with 4% paraformaldehyde using MAb 7412 against chick p75 (Tanaka et al. 1989); MAb HNK1 (Abo and Balch, 1981), and MAb JG22 (anti- β 1 integrin; Greve and Gottlieb, 1982). For analysis of melanocyte differentiation, [i]-neural plate explants were isolated from HH st. 10 quail (*Coturnix coturnix japonica*) embryos as described for equivalent chick explants (Yamada et al. 1993) and grown in vitro in collagen gels. Explants were treated with dsl-1^{myc} (3×10^{-11} M) for 48h in F12-N3 medium at which time the medium was removed, explants washed and placed in F12-N3 medium containing 10% chick embryo extract and 10% fetal calf serum for a further 72h. Dsl-1 was removed after 48h because members of the TGF β family have been found to inhibit the differentiation of neural crest cells into melanocytes (Stocker et al., 1991; Roger et al. 1992). CEE and serum were added after 48h to permit the differentiation of neural crest cells into melanocytes (Barofio et al. 1988; Maxwell et al. 1988).

Dorsal neural tube and [i]-neural plate explants grown in dsl-1^{myc} for 48h followed by defined medium lacking CEE or serum for a further 72h gave rise to few, if any, melanocytes. Thus the presence of CEE and serum appears necessary to support melanocyte differentiation under these conditions. When CEE and serum was included in the

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medium from the onset of culture, cells migrated from [i]-neural plate explants and after 120h, melanocytes were observed.

- 5 To prepare chick embryo extract, white leghorn chicken eggs were incubated for 11 days at 38°C in a humidified atmosphere. Embryos were removed and homogenized in minimal essential medium by passage through a 30 ml syringe, stirred at 40°C for 1h, and then centrifuged for
10 5h at 30,000 x g. The supernatants was collected, filtered and stored at -80°C until used.

Alkaline Phosphatase Induction in W-20-17 Cells

- 15 Induction of alkaline phosphatase activity by dsl-1 was assayed in W-20-17 cells as described (Thies et al. 1992) using recombinant human BMP-2 as a positive control.

Results

20

Isolation and Characterization of Dorsalin-1

- Degenerate oligonucleotides directed against conserved sequences present in the subfamily of TGF- β members that
25 includes the BMPs, Vg1 and dpp were used to isolate novel members of the TGF- β family (Wharton et al., 1991). Oligonucleotides were used as primers in a polymerase chain reaction (PCR) to amplify sequences derived from HH stage 16-18 (embryonic day 2.5) chick spinal cord cDNA.
30 The PCR products were cloned and 37 of 50 clones had inserts encoding Vg-1/dpp/BMP-related peptides. Although most clones encoded chick homologues of previously characterized BMP genes, one class encoded a novel sequence. A 116 bp fragment encoding this sequence was
35 used as probe to screen an E 2.5 chick spinal cord cDNA

library and to define a clone containing a 3.5 kb insert with an open reading frame that encoded a protein of 427 amino acids (Fig. 1).

5 The predicted amino acid sequence identifies this protein, dorsalin-1 (*dsl-1*), as a new member of the TGF- β superfamily. The N-terminal domain of *dsl-1* contains a stretch of hydrophobic residues that could serve as a signal sequence. A comparison of COOH-terminal 109 amino
10 acids with those of other members of this family reveals that *dsl-1* contains most of the conserved amino acids present in the other family members, including seven characteristic cysteine residues (Fig. 2A). The structure of TGF- β 2 (Daopin et al., 1992; Schlunegger and
15 Grutter, 1992) suggests that in *dsl-1*, intrachain disulfide bonds are formed between cysteines 7 and 73, 36 and 106, 40 and 108, and that cysteine 72 is involved in dimer stabilization through formation of an interchain disulfide bond. The NH² terminal domain of the *dsl-1*
20 precursor does not exhibit any significant similarity to other members of the TGF- β family.

Dsl-1 is more related to members of the Vg-1/dpp/BMP subfamily than to the TGF- β , activin or MIS subfamilies
25 (Fig. 2B). Given the high degree of sequence conservation of individual members of the BMP family identified in different species (Fig. 2), the divergence in sequence between *dsl-1* and mammalian TGF- β family members suggests that the *dsl-1* gene encodes a novel
30 member of this superfamily. The sequence of the mouse *dsl-1* gene (Cox and Basler, unpublished findings) supports this idea.

As with other family members, the conserved COOH-terminal
35 region is immediately preceded by a series of basic

residues that could serve as a site for proteolytic cleavage of the precursor protein (Celeste et al., 1990; Barr, 1991). An epitope-tagged derivative, *dsl-1^{myc}*, which contains a 10 amino acid insert derived from the human c-myc proto-oncogene (Evan et al., 1985) was generated to determine the site of cleavage of the *dsl-1* precursor. The c-myc sequence was inserted two residues upstream of the first conserved cysteine in a region of the protein that exhibits no conservation with other members of the TGF- β family (Fig. 2A). cDNAs encoding native and epitope-modified *dsl-1* were cloned into the expression vector pMT 21 and transfected separately into cos-7 cells.

Medium from cells transfected with the epitope-modified *dsl-1* construct was passed over a MAb 9E10 (Evan et al., 1985) anti c-myc affinity column. Affinity purified proteins were analyzed by gel electrophoresis, revealing a major 15 kDa band and minor bands at 45, 47 and ~60 kDa (Fig. 3A). The bands at 45 and 47 kDa correspond in size to those predicted for the unprocessed *dsl-1* protein and the 15 kDa band to that expected for a proteolytically-cleaved product. To establish the identity of the 15 kDa band and to determine the site for proteolytic cleavage of the precursor protein, the 15 kDa band was blotted onto Immobilon membranes and subjected to sequence analysis. The NH₂-terminal sequence obtained, SIGAEQKLIS (SEQ ID No. 16), corresponds to residues 319-322 of the predicted *dsl-1* sequence followed by the first 6 residues of the human c-myc epitope. This result shows that the R-S-K-R (SEQ ID No. 17) sequence at residues 315-318 is the site of proteolytic processing of the *dsl-1* precursor (arrow in Fig. 1), at least in the presence of the c-myc peptide.

To determine whether recombinant *dsl-1* secreted by cos-7 cells has BMP-like activity, a biochemical assay of osteoblast differentiation was used in which BMPs induce alkaline phosphatase activity (Thies et al. 1992).
5 Recombinant BMP-2 produced a dose-dependent increase in alkaline phosphatase activity in W-20-17 osteoblast cells over a concentration range of 10-1000 ng/nl (not shown; Thies et al. 1992). Conditioned-medium obtained from cos-7 cells transfected with *dsl-1* produced an increase
10 in alkaline phosphatase similar to that of BMP-2 at dilutions of 1:10 to 1:1000 (Fig. 3B). Moreover, medium derived from cos-7 cells transfected with *dsl-1^{myc}* cDNA, was effective as medium derived from cells transfected with unmodified *dsl-1* cDNA (Fig. 3B). In control
15 experiments, cos-7 cells were transfected with a c-myc tagged version of the *Drosophila decapentaplegic (dpp)* gene, which encodes a related TGF- β family member (Fig. 2b). Cos-7 cells do not secrete *dpp* protein (Basler, unpublished observations) and medium derived from *dpp*
20 transfectants did not induce alkaline phosphatase activity, providing evidence that cos-7 cells subjected to the same transfection protocol do not secrete a BMP-like activity (Fig. 3B). These results show that *dsl-1* can be expressed in cos-7 cells in functional form, that
25 *dsl-1* mimics the activity of BMPs in this assay and that the activity of *dsl-1* is not reduced by insertion of the c-myc peptide.

Expression of *dsl-1* RNA in the Developing Nervous System

30 *Dsl-1* mRNA was localized in developing chick embryos by in situ hybridization to examine the expression of *dsl-1* during neural development. *Dsl-1* mRNA was not expressed by cells in the neural plate (Figs. 4A,B) and first
35 appeared at the time of closure of the neural tube. At

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this stage, *dsl-1* was expressed at high levels in the dorsal third of the neural tube but was absent from more ventral regions (Figs. 4C,D). *Dsl-1* mRNA was restricted to the nervous system at this stage of development (not shown).

The restricted expression of *dsl-1* mRNA in the spinal cord persisted after the onset of neuronal differentiation (Figs. 4E-F), and by E5, the latest stage examined, the domain of expression of *dsl-1* mRNA was confined to the dorsomedial region of the spinal cord including, but not restricted to, the roof plate (Figs. 4G,H). *Dsl-1* mRNA was also expressed in dorsal regions of the hindbrain after neural tube closure (not shown). From E3 to E5, the only non-neural tissue types that expressed detectable levels of *dsl-1* mRNA were kidney and myotomal cells (not shown) although the level of mRNA expression in these tissues was much lower than that in the nervous system.

Regulation of *Dsl-1* Expression by the Notochord

The expression of antigenic markers that are restricted to dorsal neural tube cells is regulated by signals from the notochord and floor plate (Yamada et al. 1991; Placzek et al. 1991) raising the possibility that *dsl-1* mRNA expression is controlled in a similar manner. To examine this possibility, segments of stage 10 chick notochord were grafted into the lumen of the neural groove of host embryos prior to the onset of *dsl-1* mRNA expression. Embryos were incubated for a further 48h, during which time the graft was displaced dorsally, such that it is eventually located at the dorsal midline of the neural tube and spinal cord. *Dsl-1* mRNA expression, determined by in situ hybridization, was absent from the

spinal cord of embryos with dorsal notochord grafts (Figs. 5D,E) whereas the spinal cord of operated embryos at rostrocaudal levels that were not adjacent to the dorsal notochord graft exhibited the normal pattern of *dsl-1* mRNA expression (Figs. 5A,B).

To correlate changes in *dsl-1* mRNA expression with neural cell pattern, sections of operated embryos adjacent to those used for in situ hybridization were examined for expression of SC1, an immunoglobulin-like protein present on floor plate cells and motor neurons (Fig. 5C) (Tanaka and Obata, 1984; Yamada et al., 1991). In embryos in which *dsl-1* mRNA was absent from the spinal cord, SC1 expression revealed the presence of dorsal motor neurons and sometimes a floor plate at the dorsal midline of the spinal cord (Fig. 5F). Thus, dorsal notochord grafts abolish the expression of *dsl-1* mRNA and ventralize the dorsal spinal cord.

The ability of the notochord to inhibit *dsl-1* mRNA expression suggests that the notochord might normally have a role in restricting the expression of *dsl-1* within the neural tube. Elimination of ventral midline-derived signals might therefore result in an expansion in the domain of *dsl-1* expression. To test this, Hensen's node, the precursor of the notochord, was removed from stage 10 chick embryos, thus preventing the formation of the notochord and ensuring that an early source of ventral midline-derived signals (Yamada et al. 1993) is eliminated prior to neural tube formation. The spinal cords of such embryos have been shown to lack a floor plate and ventral neurons (Grabowski, 1956; Hirano et al., 1991; Darnell et al. 1992; Yamada, unpublished). In embryos from which Hensen's node had been removed, the domain of *dsl-1* mRNA expression expanded ventrally, and

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in extreme cases included the entire dorsoventral extent of the neuroepithelium (Figs. 5G,H). In a second series of experiments, the notochord was removed from the caudal region of stage 10 embryos, which were then permitted to develop for an additional 48h. At levels of the spinal cord lacking a floor plate and motor neurons, as assessed by SC1 labelling, the domain *dsl-1* expression expanded ventrally to occupy about two thirds of the spinal cord, although, the most ventral region never expressed *dsl-1* (not shown). The more limited ventral expansion of *dsl-1* observed after removal of the notochord compared with Hensen's node removal is consistent with other studies (Yamada et al. 1993) suggesting that ventralizing signals from the notochord begin to act soon after the neural plate has formed.

Taken together, these experiments suggest that the expression of *dsl-1* mRNA in ventral regions of the neural tube is normally inhibited by signals from the notochord.

Dsl-1 Regulates Neural Differentiation In Vitro

The dorsal restriction of *dsl-1* mRNA suggests two ways in which *dsl-1* protein could regulate cell differentiation along the dorso-ventral axis of the neural tube. One function of *dsl-1* could be to promote the differentiation of cell types generated in the dorsal neural tube. A second function of *dsl-1* could be to counteract the influence of ventralizing signals that derive from the notochord and floor plate. The actions of *dsl-1* on the differentiation of defined cell types in neural plate explants grown in vitro have been examined to test the possible functions of *dsl-1*. In the following sections, we provide evidence first that *dsl-1* can promote the differentiation of cells with neural crest-like

properties and second that *dsl-1* can inhibit the differentiation of motor neurons in response to inductive signals from the notochord and floor plate.

5 **Neural Crest Cell Differentiation:** Neural crest cells are generated from precursors located in the dorsal neural tube (Bronner-Fraser and Fraser, 1988). They can be identified *in vitro* by their ability to migrate from the neural tube, by their expression of several cell
10 surface markers including the HNK-1 epitope (Maxwell et al. 1988), $\beta 1$ integrin (Delannet and Duband, 1992), the low-affinity neurotrophin receptor subunit p75 (Bernd, 1985; Stemple and Anderson, 1992) and by their ability to
15 differentiate into cell types such as neurons, glial cells and melanocytes (Sieber-Blum and Cohen 1980; Baroffio et al, 1988; Stocker et al. 1991).

To examine whether *dsl-1* might regulate the differentiation or migration of neural crest cells, the
20 intermediate ([i]) region of the neural plate was isolated from stage 10 embryos and grown as explants in vitro (Yamada et al. 1993). As described (Yamada et al. 1993) few cells migrated from [i]-neural plate explants grown in isolation for 48h (Figs. 6A,G). Addition of
25 *dsl-1^{myc}* (3×10^{-11} M) for 48h resulted in a 15-fold increase in the number of cells that migrated from [i]-neural plate explants (Figs. 6B,G). To examine whether these migratory cells share surface properties with chick neural crest cells, cultures grown for 48h in the
30 presence of *dsl-1^{myc}* were labeled with monoclonal antibodies directed against HNK-1, the $\beta 1$ integrin subunit and chick p75. Over 90% of cells that had migrated from the [i]-neural plate explants in the presence of *dsl-1^{myc}* expressed HNK-1 and $\beta 1$ integrin on
35 their surface (Fig. 6D,E) and about 30% expressed p75

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(not shown). These results show that cells induced to migrate from [i]-neural plate explants have the properties of neural crest cells.

5 To determine whether the cells that are induced to migrate from [i]-neural plate explants by dsl-1 can differentiate into cell types known to derive from the neural crest, the generation of melanocytes, which can be identified unambiguously in vitro by the presence of
10 lemanin pigmentation was studied. In these experiments we used [i]-neural plate explants from quail embryos which exhibit properties in vitro similar to those of equivalently staged [i]-neural plate explants from the non-pigmented chick strain used for all other experiments
15 were used (not shown). Melanocyte differentiation from neural crest cells in vitro has been shown to require permissive factors that can be provided in the form of chick embryo extract (CEE) or serum (Baroffio et al. 1988; Maxwell et al. 1988). [i]-Neural plate explants
20 were therefore grown in dsl-1^{myc} (3×10^{-11} M) for 48h to promote the migration of cells, after which dsl-1^{myc} was removed and the medium supplemented with 10% CEE and 10% fetal calf serum and grown for a further 72h. Under these conditions, 10-15% of the cells that had emigrated
25 from [i]-neural plate explants expressed melanin pigment and exhibited dendritic morphology (Fig. 6F) indicating the presence of melanocytes. Control experiments showed that addition of CEE and serum after exposure of [i]-neural plate explants to dsl-1^{myc} for 48h did not increase
30 further the number of migratory cells (not shown). Moreover, melanocytes were not observed when [i]-neural plate explants were exposed to medium containing CEE and serum for 72h in the absence of dsl-1^{myc} (not shown). These results indicate that cells induced to migrate from
35 [i]-neural plate explants by dsl-1^{myc} can differentiate

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into at least one cell type known to derive from the neural crest.

5 In contrast to neural crest cells that derive from the dorsal neural tube d]-neural plate explants (Yamada et al. 1993), cells that had been induced to migrate from [i]-neural plate explants by $dsl-1^{myc}$ did not express neuronal markers or exhibit neuronal morphology when examined after 48h (not shown). This result suggest that
10 $dsl-1$ can promote the initial differentiation of neural crest cells from neural plate cells, but that $dsl-1$ alone does not support the subsequent differentiation of these cells into neurons.

15 The presence of migratory neural crest-like cells was also monitored to address the fate of cells in [i]-neural plate explants that have been exposed both to ventralizing signals and to $dsl-1^{myc}$. [i]-Neural plate explants grown in contact with the notochord or floor
20 plate for 48h in the presence of $dsl-1^{myc}$ ($3 \times 10^{-11}M$) exhibited a 12-15 fold increase in the number of migratory cells, similar to that observed when isolated [i]-neural plate explants were exposed to $dsl-1^{myc}$ (Fig. 6G). These cells also expressed HNK-1, $\beta 1$ integrin and
25 p75 on their surface (not shown). These findings suggest that $dsl-1^{myc}$ promotes the initial differentiation of neural crest cells in the presence of ventralizing signals from the notochord and floor plate.

30 At present, the lack of selective markers has forbidden studies of whether $dsl-1$ promotes the differentiation of other neural cell types that derive from the dorsal neural tube.

35 Regulation of Motor Neur n Differentiation: To examine

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whether *dsl-1* also influences the differentiation of ventral cell types, expression of the LIM homeodomain protein Islet-1 (Karlson et al 1990; Ericson et al. 1992), which provides a marker for the induction of motor neurons in [i]-neural plate explants in response to diffusible signal from the notochord or floor plate was monitored (Yamada et al., 1993).

[i]-Neural plate explants grown in vitro for 48h contained few (usually <5) Islet-1+ cells (Figs. 7A,B;8A,C). In contrast, [i]-neural plate explants grown in contact with notochord or floor plate exhibited a 50-100-fold increase in Islet-1+ cells (Figs. 7D,E;8A). Addition of *dsl-1^{myc}* to recombinates of [i]-neural plate with notochord or floor plate produced a concentration-dependent decrease in the number of Islet-1+ cells present in explants (Figs. 7J,K;8A,B). At concentrations of *dsl-1^{myc}* of 3×10^{-11} M or greater, the differentiation of Islet-1+ cells was suppressed by over 95% (Fig. 8B). *Dsl-1^{myc}* also abolished the expression of SC1 from regions of the [i]-neural plate explant distant from the junction with the inducing tissue (not shown) suggesting that *dsl-1^{myc}* suppresses motor neuron properties other than Islet-1. Addition of *dsl-1^{myc}* to neural plate explants grown alone did not induce Islet-1+ cells (not shown).

A truncated *dsl-1* cDNA in cos-7 cells was expressed and compared its activity with that of native *dsl-1* or *dsl-1^{myc}* to control for the presence of cos-7 cell-derived inhibitory contaminants in preparation of affinity-purified *dsl-1^{myc}*. The induction of Islet-1+ cells by floor plate was suppressed over 95% by a 1:1000 dilution of conditioned medium from cos-7 cells transfected either with unmodified *dsl-1* or with *dsl-1^{myc}* cDNAs (not shown). In contrast, medium derived from cos-7 cells expressing

the truncated *dsl-1* cDNA did not significantly reduce the number of Islet-1+ cells induced by floor plate (364±62 cells in the absence and 287±45 cell in the presence of medium containing truncated *dsl-1*, mean ±s.e.m., n=4, p>0.10).

Dsl-1 could inhibit the generation of Islet-1+ cells by preventing [i]-neural plate cells from responding to inductive signals or by inhibiting the production of this signal by the notochord and floor plate. The effects of *dsl-1^{myc}* on Islet-1+ cells in [i]-neural plate explants exposed to floor plate-conditioned medium were examined to distinguish these possibilities (Yamada et al. 1993). A 1:10 dilution of floor plate-conditioned medium produced a ~30 fold increase in the number of Isl-1+ cells (Figs. 7G,H;8C). Addition of both *dsl-1^{myc}* and floor plate-conditioned medium to neural plate explants grown alone resulted in a 76% decrease in the number of Islet-1+ cells (Fig. 8C). This result indicates that the inhibition of Islet-1+ cells results, at least in part, from a direct action of *dsl-1* on [i]-neural plate cells.

To examine whether the suppression of Islet-1+ cells is accompanied by a more general inhibition of neuronal differentiation, explants processed for Islet-1 expression were double-labelled with MAb 3A10, a general neuronal marker (Furley et al., 1990). Although the labelling of both cell bodies and axons by 3A10 made it difficult to count the number of neurons accurately, there was no obvious difference in the number of 3A10+ cells in [i]-neural plate explants exposed to concentrations of *dsl-1^{myc}* that almost completely suppressed the differentiation of Islet-1+ cells (Compare Figs. 7I and 7L). These results show that extensive neuronal differentiation still occur under conditions in

which the induction of Islet-1+ cells is suppressed.

Experimental Discussion

5

Dorsoventral patterning within the neural tube appear to begin at the neural plate stage and to involve the action of both contact-mediated and diffusible inductive signals that derive initially from the notochord and later from the floor plate. A contact-mediated signal appears to be required for floor plate differentiation whereas motor neuron differentiation can be induced by diffusible factors (Placzek et al. 1993; Yamada et al. 1993). The specification of dorsal cell types may, however, require different factors since dorsal cell types persist in the spinal cord of embryos in which the notochord and floor plate have been eliminated.

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To begin to define factors involved in specifying the fate of cells in the dorsal neural tube, a novel member of the TGF β gene family, *dorsalin-1* (*dsl*), the expression of which is restricted to the dorsal neural tube was cloned and characterized. The dorsal restriction in expression of *dsl-1* appears to be established by signals from the notochord which act on overlying neural plate cells prior to the onset of *dsl-1* transcription to prevent ventral expression of the gene after closure of the neural tube (Fig 9A). The persistence of *dsl-1* mRNA expression in the absence of the notochord and floor plate provides evidence that the expression of genes that are restricted to the dorsal neural tube is independent of ventralizing signals. Dorsal cell fates may be specified by the exposure of neural plate cells to early dorsalizing signals, perhaps from adjacent non-neural ectoderm (Takahashi et al. 1992) which induce the

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potential to express *dsl-1* and other dorsal genes.

Once the dorsal expression of *dsl-1* is established, *dsl-1* protein could function in several different ways to control cell differentiation in the neural tube. First, *dsl-1* may promote the differentiation of cell types that derive from the dorsal neural tube (Fig. 9Bi). Second, the expression of *dsl-1* could ensure that the dorsal neural tube is refractory to ventralizing signals from the notochord (Fig. 9Bii). Finally, *dsl-1* protein could diffuse and influence the fate of cells in more ventral regions of the neural tube (Fig. 9ABiii). The interactions of *dsl-1* and other factors from the dorsal neural tube with ventralizing signals from the ventral midline could, therefore control the identity of cell types and the position at which they are generated along the entire dorsoventral axis of the neural tube.

Dsl-1 May Promote Neural Crest Cell Differentiation

One function of *dsl-1* suggested by the pattern of expression of *dsl-1* mRNA could be to promote the differentiation of cell types that are generated in the dorsal neural tube. Neural crest cells constitute one of the major cell types that derive from precursors located in the dorsal neural tube. The present in vitro studies provide evidence that *dsl-1* promotes the initial differentiation of cells with neural crest-like properties from [i]-neural plate explants, but that cells exposed to *dsl-1* alone appear unable to progress to fully differentiated cell types such as neurons or melanocytes. One possible reason for this is that *dsl-1* itself may inhibit neural crest cells from further differentiation. In support of this, TGF β 1 has been shown to inhibit the

differentiation of neural crest cells into melanocytes (Stocker et al. 1991; Rogers et al. 1992) and to promote the production of extracellular matrix components such as fibronectin (Rogers et al. 1992) that can inhibit neuronal differentiation (Stemple and Anderson, 1992).
5 Alternatively other dorsally-restricted factors that are absent from [i]-neural plate explants may be required for the progression of neural crest cell differentiation.

10 TGF β 1 has also been shown to accelerate the migration of neural crest cells from premigratory regions of the neural tube (Delannet and Duband, 1992). The action of dsl-1 to promote the migration of neural crest-like cells from [i]-neural plate explants differs from this effect
15 in that cells in these explants do not give rise to neural crest cells in the absence of dsl-1 even when maintained in vitro for 96h (Yamada, unpublished observations). Nevertheless, dsl-1 may mimic the ability of TGF β 1 to accelerate neural crest migration and could
20 therefore be involved both in specifying the fate of premigratory neural crest precursors and in inducing the migration of these cells from the dorsal neural tube.

It remains unclear whether the differentiation of other
25 classes of dorsal neurons is regulated by dsl-1. Neurons with the properties of dorsal commissural neurons can differentiate in rat neural plate explants grown in isolation (Placzek et al. 1993). Thus it is possible that some dorsal cell types can differentiate
30 independently of dsl-1. Alternatively, neural plate explants grown in vitro may begin to express dsl-1 at levels sufficient to drive the differentiation of some but not all dorsal cell types.

35 Dsl-1 as an Inhibitor of Ventral Cell Type

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Differentiation

Dsl-1 suppresses the differentiation of motor neurons in [1]-neural plate explants exposed to ventralizing signals from the notochord or floor plate. This finding raises the possibility that *dsl-1* interacts with ventralizing signals to control cell fate along the dorsoventral axis of the neural tube. Although, *dsl-1* expression occurs after signals from the notochord and floor plate have begun to specify ventral cell fates (Yamada et al. 1993), its expression precedes the overt differentiation of motor neurons and other ventral neurons (Ericson et al. 1992). Indeed, the first marker of motor neuron differentiation, *Islet-1*, is not expressed until stage 15 (Ericson et al. 1992), or about 18-20h after neural tube closure and the onset of *dsl-1* expression. Thus, in the period between the initial specification and overt differentiation of neurons, *dsl-1* may accumulate to levels that are sufficient to influence neuronal differentiation.

The ability of *dsl-1* to inhibit motor neuron differentiation could be involved in preventing the generation of motor neurons and other ventral cell types in the dorsal neural tube. This presupposes that ventralizing signals from the notochord and floor plate can influence dorsal regions of the neural tube. Secreted factors from the floor plate have been shown to diffuse over long distances through the neuroepithelium (Placzek et al. 1990). Moreover the position of the ventral boundary of the domain of *dsl-1* expression suggests that signals from the notochord can influence at least two third of the neural tube. Thus, expression of *dsl-1* within the dorsal third of the neural tube could make cells in this region refractory to long range

The potential contributions of *dsl-1* to cell differentiation along the dorso-ventral axis of the neural tube will also depend on the range of action of *dsl-1* itself. Since *dsl-1* is readily secreted from cells in vitro, *dsl-1* may diffuse ventrally, beyond the domain of *dsl-1* mRNA expression, to influence the response of cells in intermediate regions of the neural tube. Again, the ability of *dsl-1* to antagonize the response of neural cells to ventralizing signals from the notochord and floor plate could be relevant both to the differentiation of motor neurons and to other ventral cell types.

Dsl-1 promotes neural crest cell migration and inhibits motor neuron differentiation in the presence of the notochord or floor plate. These findings suggest that the actions of *dsl-1* dominate over ventralizing signals. Thus, the inhibition of *dsl-1* expression from ventral regions of the neural tube that is achieved by early signals from the notochord may be necessary for the differentiation of ventral cell types. The absence of ventral cell types in the neural tube of embryos lacking a notochord could, therefore, result either from a ventral expansion in the domain of *dsl-1* expression or from the loss of ventralizing signals. However, in such operated embryos the neural tube is reduced in size (van Straaten and Hekking, 1991), thus, the death (Homma and Oppenheim, 1992) or arrested division (Placzek et al. 1993) of ventral cells could also contribute to the presence of dorsal cell types in regions of the neural tube that appear to be ventral.

Dsl-1 and the TGF β Family

In addition to *dsl-1*, several other members of the BMP (DVR) subfamily of TGF β -like genes are expressed in the embryonic nervous system. Other BMP-like proteins may therefore mimic the actions of *dsl-1* on neural cell differentiation. In preliminary studies, the induction of motor neurons was found to be also suppressed by *cos-7* cell-derived BMP-4 (Basler et al. unpublished). In the spinal cord and hindbrain, the BMP-4 (DVR-4) gene is expressed selectively by cells in the roof plate whereas in the diencephalon, the gene is found at the ventral midline (Jones et al., 1991). The expression of BMP-4 in the ventral diencephalon coincides with, and could perhaps contribute to the absence of motor neurons from the embryonic forebrain. The embryonic distribution of most other BMP genes is not known although *Vgr-1* (BMP-6/DVR-6) is expressed by cells immediately adjacent to the floor plate in the spinal cord (Jones et al., 1991) and GDF-1 appears to be expressed widely throughout the embryonic nervous system (Lee, 1990, 1991). Studies to determine whether widely distributed proteins such as GDF-1 mimic the actions of *dsl-1* will be important in evaluating the role of this gene family in neural patterning.

The involvement of *dsl-1* in the control of cell differentiation along the dorsoventral axis of the neural tube extends the range of activities described for members of the TGF β family during embryonic development. Studies in *Xenopus* embryos have provided evidence that activin can control the identity of mesodermal cell types in a concentration-dependent manner (Ruiz i Altaba and Melton, 1989; Green et al. 1992). In addition, the pattern of expression and possible functions of *dsl-1* in

the neural tube has parallels with that of the
decapentaplegic gene (*dpp*) in *Drosophila* embryonic
development (Ferguson and Anderson, 1992a,b).
Dorsoventral patterning in the early *Drosophila* embryo
5 involves a dorsal restriction of *dpp* expression (St.
Johnston and Gelbart, 1987) that is achieved by ventral-
midline derived signals that inhibit *dpp* expression
ventrally (Ray et al. 1991). Genetic inactivation of
this ventral signalling pathway or introduction of *dpp*
10 activity ventrally, changes the fate of cells along the
dorsoventral axis of the embryo (Ferguson and Anderson,
1992b). In the neural tube, the dorsal restriction of
dsl-1 mRNA by early signals from the notochord could
generate a gradient of *dsl-1* activity along the
15 dorsoventral axis of the neural tube. Alone, or in
conjunction with ventralizing signals from the notochord
and floor plate, a gradient of *dsl-1* could influence the
fate of cells according to their dorsoventral position
within the neural tube.

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